Littorina scutulata and *Littorina plena*: Sibling Species Status of Two Prosobranch Gastropod Species Confirmed by Electrophoresis

BY

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(4 Text figures)

INTRODUCTION

THE INTRODUCTION OF BIOCHEMICAL TECHNIQUES to modern taxonomic procedures has confirmed the presence of numerous sibling species whose identities were originally suspected on the basis of slight differences in morphological, cytological, or ecological characters. The most comprehensive studies using such techniques concern species complexes of *Drosophila* (HUBBY & THROCKMORTON, 1968; AYALA *et al.*, 1970; AYALA & POWELL, 1972; YANG *et al.*, 1972; COYNE, 1976). However, biochemical methods have also provided evidence of sibling species for a wide array of marine organisms, including polychaetes (GRASSLE & GRASSLE, 1976; NICKLAS & HOFFMAN, 1979), seastars (SCHOPF & MURPHY, 1973), sea cucumbers (MANWELL & BAKER, 1963), barnacles (HEDGECOCK, 1979; DANDO & SOUTHWARD, 1980), fiddler crabs (SALMON *et al.*, 1979), limpets (MURPHY, 1978), and sea anemones (BUCKLIN & HEDGECOCK, 1981). In each instance, biochemical data have indicated genetic isolation and have provided diagnostic characters for distinguishing between closely-related sibling species.

Snails currently classified as *Littorina scutulata* Gould, 1849, are upper littoral gastropods of the west coast of North America. They are abundant residents on rocks and pilings in sheltered bays as well as on rocky shores of the exposed open coast. MURRAY (1979) has shown that a dichotomy exists in the reproductive biology of *L. scutulata*. A dimorphism of genitalia occurs among males, and individual females produce one of two morphologically distinct types of planktonic egg capsules, differing in size, in shape, in numbers of eggs per capsule, and in the location of the hatching pore through which swimming veligers emerge. Developmental rates within the two types of egg capsules also appear to differ, further suggesting that the taxon *L. scutulata* is actually a complex of two morphologically similar species.

The purpose of this study is to assess the degree of genetic and reproductive separation between the two morphological forms of *Littorina scutulata*. The techniques of gel electrophoresis are employed to confirm their taxonomic status as separate species. We suggest that the name *Littorina plena* Gould, 1849, be revived for the second sibling species.

MATERIALS AND METHODS

Populations Studied

Adult snails belonging to the *Littorina scutulata* species complex were collected at random from rocky shores at 15 localities (Figure 1). These samples were taken at irregular intervals from the summer of 1979 through the summer of 1980. Samples from the following five sites in California were selected for detailed study by horizontal starch gel electrophoresis: Newport Beach, Orange County (NB); Shell Beach, San Luis Obispo County (SLO); Bodega Bay (BB) and Sea Ranch (SR), Sonoma County; and Point Arena, Mendocino County (PA). Other observations were made on snails from additional sites in California: Bird Rock, San Diego County (SD); Laguna Beach, Orange County (LB); Goleta, Santa Barbara County (SB); Pacific Grove, Monterey County (MON); Berkeley Marina inside San Francisco Bay (SF); Dillon Beach, Marin County (DB); and Trinidad, Humboldt County (HUM). Collections were also made at Cape

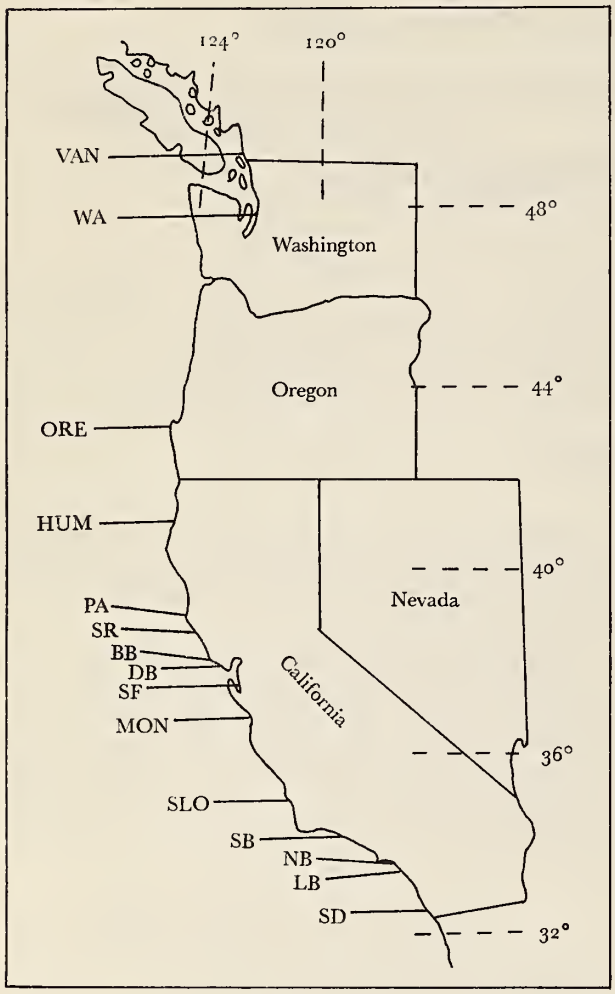

Figure 1

Map of California, Oregon, and Washington showing the approximate locations of sampling sites

Arago, Oregon (ORE), at several sites within Puget Sound, Washington (WA), and at Vancouver, Canada (VAN).

Snails were either transported directly or sent live by first class mail to the University of California Bodega Marine Laboratory. Individuals were separated by sex and by reproductive type (species) according to the characters described by Murray (1979). Male snails were identified by penis morphology and female snails by the type of egg capsules produced once isolated in individual containers. Only identifiable snails (reproductively active females and males with shell lengths greater than 4 mm) were used in the electrophoretic study.

Sample Preparation and Assay Technique

Snails selected for electrophoresis were maintained in running seawater without food for at least 48 hours after collection, and then were kept frozen at −80° C if not used immediately. Animals were removed whole from their shells, and the tissues were homogenized with an approximately equal volume of 0.5 M tris-HCl buffer (pH 7.1) while submerged in an ice-water bath. A small amount of the homogenate was absorbed with Whatman No. 1 filter paper wicks for insertion into starch gels.

Electrophoretic procedures were carried out essentially as described by Ayala *et al.* (1972). Samples of 23 snails were run in each gel along with two control samples. Controls consisted of individuals whose isozyme mobilities were previously determined. Each gel included snails of both reproductive morphologies.

The following five buffer systems were used to separate the ten enzymes assayed in all populations: (A) Discontinuous, tris-citrate electrode buffer, pH 8.65, borate (NaOH) gel buffer, pH 8.1 (Ayala *et al.*, 1973); (B) Continuous, tris-borate-EDTA electrode and gel buffer, pH 9.1 (Ayala *et al.*, 1973); (C) Continuous, tris-citrate-EDTA electrode buffer, pH 7.0, with a 15-fold dilution of the electrode buffer for the gel buffer (Ayala *et al.*, 1973); (D) Continuous, citric acid-phosphate electrode and gel buffer, pH 7.0 (Shaw & Prasad, 1970); (E) Discontinuous, LiOH-boric acid electrode buffer, pH 8.1, tris-boric acid-citric acid LiOH gel buffer, pH 8.2 (Selander *et al.*, 1971). The buffer system used for each enzyme assay is specified in Table 1.

Enzyme-staining assays were conducted as described by Ayala *et al.* (1972, 1973, 1974) and by Tracey *et al.*

Table 1

Enzymes assayed in *Littorina scutulata* and *Littorina plena* populations.

Enzyme	Abbreviation	Buffer system
Acid phosphatase	ACPH-2	A
Esterase	EST	E
Glutamate oxaloacetate transaminase	GOT	D
Leucine amino peptidase	LAP-1	A
Lactate dehydrogenase	LDH	B
Mannose-6-phosphate isomerase	MPI	D
6-Phosphogluconate dehydrogenase	6-PGDH	C
Phosphoglucose isomerase	PGI	E
Phosphoglucomutase	PGM	C
Sorbitol dehydrogenase	SDH	D

Table 2

Allelic frequencies of the ten loci examined for populations of *Littorina scutulata* and *Littorina plena*. The value *n* equals the number of alleles sampled in each population.

		Littorina scutulata populations					*Littorina plena* populations				
Locus	Allele	PA	SR	BB	SLO	NB	PA	SR	BB	SLO	NB
Acph-2	*n*	*18*	*18*	*20*	*20*	*20*	*22*	*20*	*20*	*20*	*20*
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Est	*n*	*18*	*20*	*64*	*20*	*20*	*18*	*18*	*32*	*20*	*26*
	98	0.39	0.65	0.44	0.40	0.63	—	0.11	—	0.10	0.08
	100	0.61	0.35	0.56	0.60	0.31	0.05	0.45	0.56	0.40	0.42
	102	—	—	—	—	0.06	0.77	0.33	0.44	0.50	0.50
	103	—	—	—	—	—	0.18	0.11	—	—	—
Got	*n*	*18*	*32*	*20*	*20*	*34*	*22*	*28*	*20*	*20*	*32*
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lap-1	*n*	*18*	*20*	*40*	*20*	*20*	*22*	*20*	*42*	*20*	*20*
	100	0.78	0.50	0.55	0.70	0.55	0.77	0.60	0.38	0.65	0.45
	101	0.22	0.50	0.45	0.30	0.45	0.23	0.40	0.62	0.35	0.55
Ldh	*n*	*18*	*36*	*46*	*20*	*32*	*22*	*20*	*24*	*20*	*16*
	98	0.11	—	—	—	—	—	—	—	—	—
	100	0.89	0.97	1.00	1.00	1.00	0.18	0.20	—	0.45	0.31
	103	—	0.03	—	—	—	0.82	0.80	1.00	0.55	0.69
Mpi	*n*	*18*	*20*	*10*	*20*	*40*	*22*	*20*	*10*	*20*	*40*
	98	—	—	—	0.20	—	—	—	—	0.10	0.25
	100	1.00	1.00	1.00	0.75	1.00	1.00	1.00	1.00	0.55	0.75
	102	—	—	—	0.05	—	—	—	—	0.35	—
6-pgdh	*n*	*18*	*32*	*64*	*20*	*34*	*22*	*18*	*66*	*20*	*24*
	94	—	0.03	—	—	0.24	—	—	—	—	—
	96	—	—	—	—	—	—	—	0.03	—	—
	100	1.00	0.94	0.97	1.00	0.76	0.91	0.78	0.73	0.70	0.79
	103	—	0.03	0.03	—	—	0.09	0.22	0.24	0.30	0.21
Pgi	*n*	*18*	*36*	*64*	*20*	*34*	*22*	*28*	*66*	*20*	*32*
	90	0.05	—	0.01	—	0.06	—	—	—	—	—
	93	—	0.14	—	0.05	0.03	—	—	—	—	—
	96	0.17	0.11	0.19	0.25	0.15	—	—	—	—	—
	100	0.78	0.47	0.50	0.45	0.38	0.05	—	—	0.05	—
	103	—	0.28	0.19	0.25	0.32	0.63	0.68	0.74	0.75	0.72
	106	—	—	0.11	—	0.06	—	—	—	—	—
	107	—	—	—	—	—	0.27	0.32	0.24	0.20	0.25
	110	—	—	—	—	—	0.05	—	0.02	—	0.03
Pgm	*n*	*18*	*34*	*64*	*20*	*34*	*22*	*28*	*66*	*20*	*32*
	97	—	0.15	—	0.05	0.03	—	—	—	—	—
	100	0.39	0.53	1.00	0.65	0.73	—	0.10	—	—	—
	102	0.57	0.26	—	0.30	0.21	0.64	0.75	0.70	0.75	0.78
	105	0.04	—	—	—	0.03	0.23	0.04	0.26	0.20	0.09
	107	—	0.06	—	—	—	—	0.04	—	0.05	0.13
	110	—	—	—	—	—	0.13	0.07	0.04	—	—
Sdh	*n*	*18*	*34*	*14*	*20*	*20*	*22*	*22*	*24*	*20*	*20*
	94	—	0.03	—	—	—	—	—	—	—	—
	100	1.00	0.97	1.00	0.90	0.85	0.14	0.18	0.29	0.30	0.40
	102	—	—	—	0.10	—	—	—	—	—	—
	107	—	—	—	—	0.15	0.86	0.82	0.71	0.70	0.60

(1975) with the following additions and modifications. *Sorbitol dehydrogenase*: 1.0 g D-sorbitol, 20 mg NAD, 25 mg NBT, 100 ml 0.05M tris-HCl buffer (pH 8.0), 5 mg PMS. *Mannose-6-phosphate isomerase*: 60 mg mannose-6-phosphate, 25 mg NADP, 20 mg MTT, 40 units G-6-PDH, 30 units PGI, 100 ml 0.5M tris-HCl buffer (pH 8.0), 5 mg PMS.

The abbreviations for the enzymes, as given in Table 1, are used to indicate the corresponding gene loci when the abbreviations are *italicized*. When more than one zone of activity exists for a given enzyme, a suffix has been added to the abbreviation to designate which zone is being considered. The zone of activity with the least migration is designated 1, the next is designated 2, and so on. The esterase zymogram has numerous zones of activity and only the zone with the greatest migration is considered in this study. For each locus, the most common allele in the Bodega Bay population of *Littorina scutulata* has been arbitrarily labelled 100. The other alleles are labelled in relation to this standard by addition or subtraction from 100 of the number of millimeters by which their migration differs from the standard.

Allelic frequencies in different populations of the two species of *Littorina* were compared by chi-square analyses of the observed allele numbers. Classes of alleles were combined whenever expected allele numbers were less than three, and an adjusted chi-square was calculated for comparisons with one degree of freedom. Results were further analyzed by measuring the degree of genetic similarity between populations, using the statistic *I* as defined by Nei (1972). Values of *I* range from 0 to 1, where a value of 1 indicates genetic identity.

RESULTS

Electrophoresis

The results of the ten enzyme assays for the two sibling species are summarized in Table 2. Sample sizes and allelic frequencies for each population are shown, where sample size is the number of alleles assayed. Of the ten loci examined, two (*Acph-2* and *Got*) are monomorphic, *i.e.*, all individuals possess the same allele. The remaining eight loci are polymorphic with the number of alleles per enzyme ranging from two in *Lap-2* to eight in *Pgi*.

The chi-square analyses indicate that *Littorina scutulata* and *L. plena* do, indeed, have characteristic allelic frequencies. The differences between sympatric populations of the two species are all highly significant ($P<0.01$) at the *Est, Ldh, Pgi, Pgm,* and *Sdh* loci. The Newport Beach populations also differ significantly ($P<0.01$) at the *Mpi* locus, and populations at three out of five localities tested show some difference at the *6-pgdh* locus ($P<0.05$). Over all localities, the differences between *L. scutulata* and *L. plena* are significant ($P<0.05$) in all between-site comparisons at the *Est, Ldh, Pgi,* and *Sdh* loci. The allelic frequencies of the two species also differ in a statistically significant manner in 18 of 20 between-site comparisons at the *Pgm* locus, in 5 of 20 comparisons at the *6-pgdh* locus, and in 5 of 20 comparisons at the *Mpi* locus ($P<0.05$).

Genetic similarities between populations (Nei's *I*) are shown in Table 3 for all possible pairwise combinations. The average genetic similarity between the populations of *Littorina scutulata* is 0.962 ± 0.025. The average genetic

Table 3

Genetic similarities *(I)* between pairs of *Littorina* populations.

	Littorina scutulata populations				*Littorina plena* populations				
	SR	BB	SLO	NB	PA	SR	BB	SLO	NB
Littorina scutulata									
PA	0.9581	0.9413	0.9694	0.9010	0.6787	0.7249	0.6728	0.7421	0.7453
SR	—	0.9720	0.9736	0.9852	0.6740	0.7490	0.6837	0.7417	0.7586
BB	—	—	0.9752	0.9764	0.6207	0.6784	0.6314	0.6848	0.6978
SLO	—	—	—	0.9668	0.6650	0.7222	0.6815	0.7566	0.7566
NB	—	—	—	—	0.6858	0.7366	0.6816	0.7481	0.7544
Littorina plena									
PA	—	—	—	—	—	0.9640	0.9404	0.9363	0.9421
SR	—	—	—	—	—	—	0.9773	0.9554	0.9737
BB	—	—	—	—	—	—	—	0.9338	0.9704
SLO	—	—	—	—	—	—	—	—	0.9772

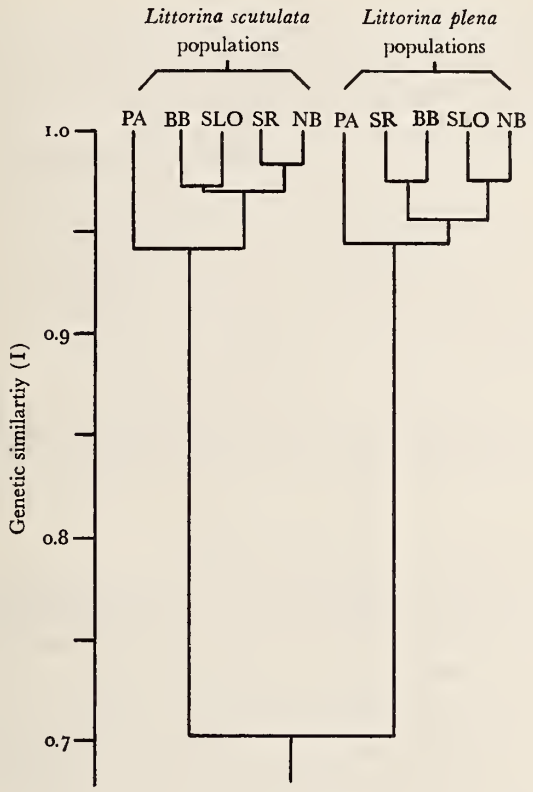

Figure 2

Dendrogram expressing levels of genetic similarity among *Littorina scutulata* and *Littorina plena* populations (UPGM cluster analysis)

similarity is likewise high for populations of *L. plena.* (0.957±0.018). Measures of genetic similarity between populations of the two species, however, are consistently lower, averaging 0.707±0.058. The genetic similarities between all pairs of populations have also been converted into a dendrogram (Figure 2) using an unweighted pair group method of cluster analysis (Sokal & Sneath, 1963). Clearly, the ten populations fall into two groups, one consisting of the five *L. scutulata* populations and the other composed of the five *L. plena* populations. The degree of genetic differentiation between the two species is far greater than that within either.

Morphology

Consistent specific differences in the general morphology of egg capsules and penes exist between *Littorina scutulata* and *L. plena* at all fifteen localities studied (Figure 3). Female *L. plena* produce planktonic egg capsules with two outer rims of nearly equal diameters, while males possess an attenuate penis with one large papilla and an elongated tip. Female *L. scutulata* spawn egg capsules with two rims of very different diameters, while males possess a somewhat truncate penis lacking papillae.

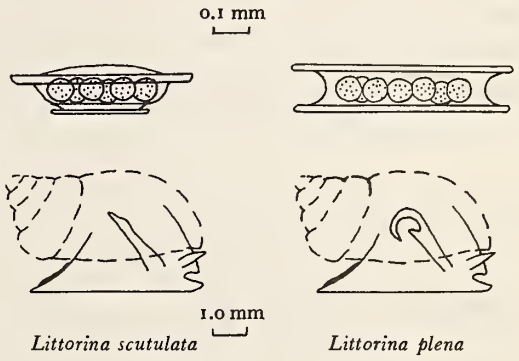

Figure 3

The planktonic egg capsule and penis morphology of *Littorina scutulata* and *Littorina plena*

The shell morphology of both species appears to vary with both habitat and geographic location. Furthermore, intraspecific variation often results in considerable interspecific overlap in any one shell character for sympatric populations of the two species. *Littorina plena* from exposed rocky shores near Bodega Bay become mature at shell lengths of 2-3 mm and grow to a maximum length of 11 mm. Most individuals have shells with 3-4 whorls. The interior of the aperture usually displays an amber band curving inward near the base of the shell. *L. scutulata* from the same shores become mature at lengths of 4-7 mm and reach lengths of 17 mm. Individuals possess shells with approximately 4-5 whorls that usually lack the amber band seen in *L. plena.* Shell color in both species is highly variable, ranging from black or dark purple to gray, green, or light brown. However, the shells of *L. scutulata* do tend to have more tessellations than those of *L. plena.*

The radular morphologies of *Littorina scutulata* and *L. plena* are typical of all Littorinidae (Figure 4). Radulae of both species stained in fuchsin and examined under a light microscope show numerous transverse rows of teeth, each row consisting of a single central or rachidian tooth flanked on each side by one lateral and two marginal teeth. Although the interspecific overlap is again large,

Table 4

Length/width ratios of the rachidian tooth of *Littorina scutulata* and *Littorina plena* from Bodega Bay. Approximately 30 measurements were made with an ocular micrometer at 400× and then averaged for each replicate radula. Length/width ratios were statistically independent of shell length in each species.

Species	*n*	Range of tooth lengths (microns)	Range of tooth widths (microns)	Length/width ratio ± one standard deviation
Littorina scutulata	20	37.1 - 52.5	42.5 - 63.0	0.868 ± 0.053
Littorina plena	20	10.6 - 34.3	10.6 - 32.0	0.990 ± 0.084

Wilcoxon's two sample test: *Littorina scutulata ratio* ≠ *Littorina plena* ratio; $P \ll 0.001$.

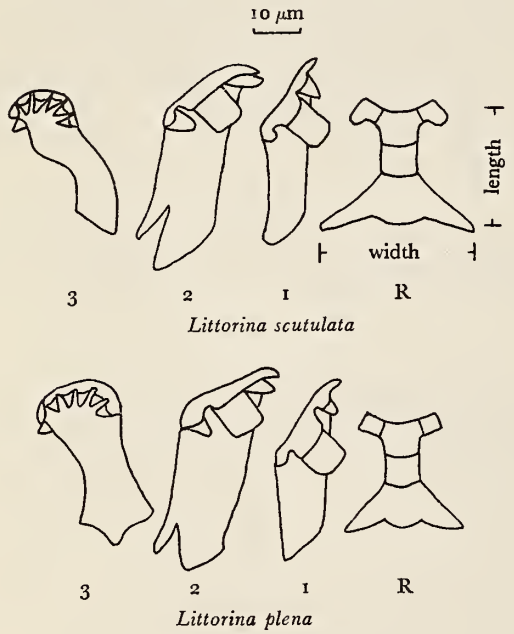

Figure 4

Radular teeth from one side of a transverse row
R – rachidian; 1 – lateral; 2 – inner marginal
3 – outer marginal

Bodega Bay populations of the two species differ significantly in the length to width ratio of the rachidian tooth (Table 4).

Taxonomy

Gould (1849) described three species of *Littorina* from the west coast of North America which were subsequently synonymized under the name *L. scutulata* by Carpenter (1864). The type specimens for the three species kept at the United States National Museum of Natural History were examined to clarify their taxonomic status.

Type Material and Type Localities

Littorina scutulata Gould: (Puget Sound, Washington)
Holotype USNM 5640: length 12.43 mm, width 8.26 mm
Paratype USNM 612308: length 8.97 mm, width 5.86 mm
Littorina plena Gould: (San Francisco, California)
Lectotype USNM 5635: length 8.53 mm, width 5.42 mm
Paralectotypes USNM 677096: both males; length 8.34 mm, width 5.55 mm; length 6.42 mm, width 4.27 mm
Littorina lepida Gould: (Puget Sound, Washington)
Lectotype USNM 5637, length 9.11 mm, width 6.12 mm
Paralectotype USNM 677095, length 8.11 mm, width 5.15 mm

Measurements are given with shell width perpendicular to shell length.

The holotype of *Littorina scutulata* is large and highly tessellated. This specimen probably represents a group distinct from *L. plena,* and we suggest that *L. scutulata* Gould, 1849, be retained as a valid name for the species as established in this investigation.

The paralectotypes of *Littorina plena* contained intact bodies within the shells. The bodies were rehydrated, removed, and positively identified as male *L. plena* by the morphology of their penes. On the basis of this identification, we propose that the species name *L. plena* Gould, 1849, be resurrected to define the second sibling species (even though other type material may prove to be a mixture of species).

At this time, the type material of *Littorina lepida* cannot be reliably assigned to either *L. scutulata* or *L. plena.*

Voucher specimens of *Littorina scutulata* have been obtained from the exposed rocky shores northwest of Horseshoe Cove on the biological reserve of the University of California, Bodega Bay, California. In addition to the Bodega Bay location, voucher specimens of *L. plena* have been obtained from the rip-rap on the south side of

the Berkeley Marina and from rocky outcrops at the north end of Baker's Beach, San Francisco, California. These specimens have been deposited at the National Museum of Natural History (USNM), the California Academy of Sciences (CASIZ), and the Natural History Museum of Los Angeles County (LACM).

Littorina scutulata voucher specimens and locality:
USNM 803490, CASIZ 024458, LACM 67755: Bodega Bay, California (38°19'00" N; 123°04'16" W)
Littorina plena voucher specimens and localities:
USNM 803491, CASIZ 024456, LACM 67756: Bodega Bay, California (38°19'00" N; 123°04'16" W)
USNM 803492, CASIZ 024457, LACM 67757: Berkeley, California (37°51'18" N; 122°18'50" W)
USNM 803493, CASIZ 024455, LACM 67758: San Francisco, California (37°48'21" N; 122°28'40" W)

DISCUSSION

The taxonomy of several *Littorina* species has been recently revised to include the presence of additional, morphologically similar species. Sympatric sibling species of *Littorina* are now recognized on the Hawaiian Islands (Whipple, 1965), among the West Indian fauna (Borkowski & Borkowski, 1969), and on British shores (Heller, 1975; Hannaford Ellis, 1979). *Littorina scutulata* and *L. plena* represent still another example of co-existing species of littoral gastropods possessing very similar morphological and ecological attributes.

The electrophoretic data presented in this study indicate reproductive and genetic separation, if not total isolation, between *Littorina scutulata* and *L. plena.* Within either species, very little genetic differentiation appears to have occurred between populations as far apart as San Diego and Point Arena. Interspecific comparisons, however, consistently show significant differences in allelic frequencies between *L. scutulata* and *L. plena,* at levels exceeding the genetic differentiation measured by Wilkins & O'Regan (1980) for three species of the British *L. saxatalis* species complex and by Ward & Warwick (1980) for *L. arcana* Hannaford Ellis, 1978, and *L. rudis* (Maton, 1797). Such biochemical evidence indicates that *L. scutulata* and *L. plena* are two separate reproductive and genetic entities worthy of species status.

Various aspects of the reproductive biology of *Littorina* are often species-specific traits, and differences in these traits can be strong evidence of separate sibling species. Mode of reproduction (Heller, 1975), morphology of genitalia (Whipple, 1965; Heller, 1975; Goodwin & Fish, 1977), and characteristics of spawn (Whipple, 1965; Borkowski & Borkowski, 1969) are used to distinguish between snails whose identities based on shell morphology are ambiguous. Murray (1979) notes for *L. scutulata* and *L. plena* that females of each species spawn characteristic egg capsules; in addition, males display differences in penis morphology, differences similar to those which Heller (1975) suggests may be of importance in species recognition and ethological isolation of British *Littorina.* Although reproductive attributes have been questioned as taxonomic characters in certain instances (Borkowski, 1975; Raffaelli, 1979; Caugant & Bergerard, 1980), the agreement of the biochemical data in this investigation with the dichotomy described by Murray (1979) indicates that the reproductive distinctions between *L. scutulata* and *L. plena* are reliable species discriminators.

In response to environmental clines, many species of *Littorina* exhibit considerable variation in morphological features (Strusaker, 1968; Newkirk & Doyle, 1975; Hylleberg & Christensen, 1977) to the extent that the taxonomic status of some populations has been in doubt (Colman, 1932; Hughes, 1979; Raffaelli, 1979). *Littorina scutulata* and *L. plena* occupy a diversity of habitats ranging in degree of exposure from protected bays and estuaries to the exposed open coast, and both species show substantial intra-habitat as well as inter-habitat variation in shell and radular characters. Reliable separation of the two species on the basis of these morphological traits is likely to depend upon the particular populations examined and may involve a combination of several shell or radular characters, or both.

In such cases where regional and intra-population variation in morphology are great, the application of electrophoretic techniques produces results which are less affected by factors other than the genotype of the individual. In this study, the biochemical genetic evidence supports subtle morphological differences as well as the reproductive distinctions previously detailed by Murray (1979) as indicators of two biological species. These results confirm the presence of the sympatric sibling species, *Littorina scutulata* and *L. plena.*

ACKNOWLEDGMENTS

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